HYBRIDIZATION-BASED BIOSENSOR CONTAINING HAIRPIN PROBES AND USE THEREOF

This application claims the benefit of U.S. Provisional Patent Application 60/437,780 filed January 2, 2003, which is hereby incorporated by reference in its entirety.

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FIELD OF THE INVENTION

The present invention relates to hybridization-based biosensors containing hairpin probes and their use in identifying target nucleic acids in samples.

BACKGROUND OF THE INVENTION

Recent intense interest in the use of rapid genetic analysis as a tool for understanding biological processes (Wodicka et al., *J. Nat. Biotechnol.* 15:1359-1367 (1997); Iyer et al., *Science* 283:83-87 (1999)), in unlocking the underlying molecular causes of disease, and in the development of biosensors, has led to a need for new sensitive and arrayable chip-based analytical tools. Of high importance is the need for techniques that do not require labeling of the target sample (Sando et al., *J. Am. Chem. Soc.* 124:2096-2097 (2002), since that increases the time, cost, and potential for error inherent in the analysis. In the context of solution-phase assays, the molecular beacon concept has proven itself to be both sensitive and reliable (Broude, *Trends Biotech.* 20:249-256 (2002); Dubertret et al., *Nature Biotech.* 19:365-370 (2001)). Molecular beacons consist of a DNA hairpin functionalized at one end with a fluorophore, and at the other with a quenching agent (Tyagi et al., *Nat. Biotechnol.* 14:303-308 (1996); Joshi et al., *Chem. Commun.* 1(6):549-550 (2001)). In the absence of the target DNA sequence, the quencher is brought in close proximity to the fluorophore, and no signal is generated. Addition of the target sequence leads to hairpin unfolding, concomitant duplex formation, and signal generation.

Although a few reports of surface-immobilized molecular beacons have appeared in the literature (Fang et al., *J. Am. Chem. Soc.* 121:2921-2922 (1999);

Wang et al., *Nucl. Acids. Res.* 30:e61 (2002)), it is believed that these approaches employ an attached single molecule as quencher, while the material on (or in) which the hairpin is immobilized serves only a passive role. As part of a general program aimed at developing "label-free" optical biosensors (Chan et al., *J. Am. Chem. Soc.* 123:11797-11798 (2001)), it was of interest, therefore, to investigate whether the substrate material itself could be used as a quenching agent.

When attempting to adapt the work of Dubertret et al. (*Nature Biotech.* 19:365-370 (2001)) by attaching fluorophore-functionalized DNA hairpins to a flat gold surface rather than a gold nanoparticle, as described by Dubertret et al., the inventors of the present application obtained a device that was not functional, presumably because of steric crowding. The gold nanoparticles used by Dubertret et al. contained only a single hairpin per particle; whereas multiple hairpins were bonded to the flat gold surface.

The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

A first aspect of the present invention relates to a sensor chip that includes: a fluorescence quenching surface; a first nucleic acid molecule (i.e., as a probe) that contains first and second ends with the first end bound to the fluorescence quenching surface, a first region, and a second region complementary to the first region, the first nucleic acid molecule having, under appropriate conditions, either a hairpin conformation with the first and second regions hybridized together or a non-hairpin conformation; and a first fluorophore bound to the second end of the first nucleic acid molecule. When the first nucleic acid molecule is in the hairpin conformation, the fluorescence quenching surface substantially quenches fluorescent emissions by the first fluorophore; and when the first nucleic acid molecule is in the non-hairpin conformation (i.e., in the presence of a target nucleic acid molecule), fluorescent emissions by the fluorophore are substantially free of quenching by the fluorescence quenching surface.

A second aspect of the present invention relates to a biological sensor device that contains a sensor chip according to the first aspect of the present invention,

a light source that illuminates the sensor chip at a wavelength suitable to induce fluorescent emissions by the first fluorophore, and a detector positioned to detect fluorescent emissions by the first fluorophore.

A third aspect of the present invention relates to nucleic acid molecules that can be used as probes on sensor chips in accordance with the first aspect of the present invention. The nucleic acid probe includes first and second ends, the first end being modified for coupling to a surface and the second end being bound to a fluorophore, the nucleic acid probe further including a first region, and a second region complementary to the first region, wherein, under appropriate conditions, the nucleic acid probe has either a hairpin conformation with the first and second regions hybridized together or a non-hairpin conformation, with one or both of the first and second regions being adapted for hybridization to a target nucleic acid molecule.

A fourth aspect of the present invention relates to a method of detecting the presence of a target nucleic acid molecule in a sample. This method of the invention is carried out by: exposing the sensor chip according to the first aspect of the present invention to a sample under conditions effective to allow any target nucleic acid molecule in the sample to hybridize to at least a portion of the first and/or second regions of the first nucleic acid molecule; illuminating the sensor chip with light sufficient to cause emission of fluorescence by the first fluorophore; and determining whether or not the sensor chip emits fluorescent emission of the first fluorophore upon said illuminating, wherein fluorescent emission by the sensor chip indicates that the first nucleic acid molecule is in the non-hairpin conformation and therefore that the target nucleic acid molecule is present in the sample.

A fifth aspect of the present invention relates to a method of genetic screening that is carried out by performing the method according to the fourth aspect of the present invention using a sensor chip having a first nucleic acid molecule with the first and/or second region thereof specific for hybridization with a first genetic marker.

A sixth aspect of the present invention relates to a method of detecting the presence of a pathogen in a sample that includes performing the method according to the fourth aspect of the present invention with a sensor chip having a first nucleic acid molecule with at least a portion of the first and/or second region thereof specific for hybridization with a target nucleic acid molecule of a pathogen.

A seventh aspect of the present invention relates to a method of making a sensor chip of the present invention. This method is carried out by: providing a fluorescence quenching surface; exposing the fluorescence quenching surface to a plurality of first nucleic acid molecules each comprising first and second ends with the first end being modified for coupling to the fluorescence quenching surface, a first region, and a second region complementary to the first region, and each first nucleic acid molecule having, under appropriate conditions, either a hairpin conformation with the first and second regions hybridized together or a non-hairpin conformation; and exposing the fluorescence quenching surface to a plurality of spacer molecules each including a reactive group capable of coupling to the fluorescence quenching surface, whereby the plurality of spacer molecules, when bound to the fluorescence quenching surface, inhibit interaction between adjacent first nucleic acid molecules bound to the fluorescence quenching surface (i.e., thereby minimizing background fluorescence by the sensor chip in the absence of target nucleic acid molecules).

The present invention allows for the simple construction of single or arrayed sensors in a convenient format. In particular, the use of secondary sensor agents is obviated by the presence of the quenching agent and the fluorescent agent in a single structural arrangement. Following one or more hybridization procedures, presence or absence of target nucleic acids is identified by the presence of a fluorescent signal emitted by a fluorophore bound to the hairpin probe that is tethered to the quenching substrate. The sensor chips and sensing devices of the present invention allow for a visual inspection by a person or instrument to see one or more colors, allowing for the simple detection of even low levels of target nucleic acids. These results could not be achieved with the molecular beacons employed, for example, by Dubertret et al. (*Nature Biotech.* 19:365-370 (2001), which is hereby incorporated by reference in its entirety). Moreover, the methods and devices of the present invention can also take advantage of surface enhancement of the electric field caused by the fluorescence quenching surface to attain orders of magnitude more signal per photon than that achieved by Dubertret et al.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a sensor chip of the present invention. A hairpin nucleic acid molecule is immobilized at one end thereof to a fluorescent quenching surface, and the other end thereof has attached thereto a fluorophore. In the hairpin conformation, the fluorophore is in sufficiently close proximity to the fluorescent quenching surface such that fluorescent emissions of the fluorophore are quenched. In the presence of a target nucleic acid molecule, the hairpin conformation is lost, resulting in fluorescent emissions that are no longer quenched by the fluorescent quenching surface.

Figure 2 illustrates one particular embodiment of the sensor chip, where two or more nucleic acid hairpin probes are bound to the fluorescent quenching surface so that they are present in discrete locations.

Figure 3 illustrates another embodiment of the sensor chip, where two or more nucleic acid hairpin probes are bound to the fluorescent quenching surface so that they are co-localized. Different fluorophores having distinct fluorescent emissions distinguish one probe from another.

Figure 4 is a schematic showing a biological detection device according to one embodiment of the present invention, which includes, *inter alia*, an inverted fluorescence microscope equipped with a liquid nitrogen cooled charge coupled device (CCD).

Figure 5 illustrates the predicted folding structure of the nucleotide sequence for hairpin H1 (SEQ ID NO: 1), which was designed to incorporate portions of the *Staphylococcus aureus FemA* methicillin-resistance gene (Berger-Bachi et al., *Mol. Gen. Genet.* 219:263-269 (1989); Genbank accession X17688, each of which is hereby incorporated by reference in its entirety). The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety) and later confirmed by melting experiments.

Figure 6 illustrates the predicted folding structure of the nucleotide sequence for hairpin H2 (SEQ ID NO: 2), which was designed to incorporate portions of the *Staphylococcus aureus mecR* methicillin-resistance gene (Archer et al., *Antimicrob. Agents. Chemother.* 38:447-454 (1994), which is hereby incorporated by

reference in its entirety). The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety) and later confirmed by melting experiments.

Figures 7A-B illustrate the secondary structure of hairpin HP1 alone (7A) and the hybrid HP1-T1 (7B). HP1 corresponds to SEQ ID NO: 7, which is targeted to a complementary sequence T1 (SEQ ID NO: 11) that is substantially homologous to *Bacillus anthracis pag*. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figures 8A-B illustrate the secondary structure of hairpin HP2 alone (8A) and the hybrid HP2-T2 (8B). HP2 corresponds to SEQ ID NO: 5, which is targeted to a complementary sequence T2 (SEQ ID NO: 12) that is substantially homologous to *Bacillus anthracis pag*. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figure 9 illustrates the secondary structure of a hairpin probe corresponding to SEQ ID NO: 3, which is targeted to the *Exophiala dermatitidis* 18S ribosomal RNA. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figure 10 illustrates the secondary structure of a hairpin probe corresponding to SEQ ID NO: 4, which is targeted to the *Trichophyton tonsurans* 18S ribosomal RNA. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figures 11A-D are graphs illustrating the results of thermal melts of H1 alone (11A), H1 and T1 together (11B), H2 alone (11C) and H2 and T2 together (11D). All thermal melts were conducted on a Gilford spectrophotometer, with the oligonucleotide dissolved in 0.5 M NaCl Buffer. Comparing Figures 7A-B, the measured melting temperature of H1 is about 69 °C. Comparing Figures 7C-D, the measured melting temperature of H2 is about 58 °C.

Figures 12A-B illustrate the output of the CCD for binding to hairpin probe H1. Figure 12A shows the CCD image pre-hybridization of the target nucleic acid molecule. Figure 12B shows the CCD image post-hybridization. A clear signal, distinct of background, is obtained following hybridization of T1 to H1.

Figures 13A-B are graphs illustrating the hybridization-dependent fluorescence efficiency of the sensors containing nucleotide sequences H1 (Figure 13A) and H2 (Figure 13B). The fluorescence efficiency was determined using CCD images with dark counts subtracted and all pixels binned in the vertical direction, for both sequences before and after hybridization. The integration time was 10 seconds.

Figures 14A-D illustrate the selectivity of hybridization assays. Figures 14A-C are digital images showing post-immobilization of sequence H1 (14A), post treatment with 1.38 μM of hybridizing target sequence T1 (14B), and post treatment with 1.38μM salmon sperm DNA (14C). Figure 14D is a graph illustrating the following binned CCD intensity images: curves (a) and (d) show fluorescence pre-immobilization of sequence H1, curves (b) and (e) show fluorescence post-immobilization of sequence H1, curve (c) shows fluorescence post treatment with 1.38 μM of hybridizing target sequence T1, and curve (f) shows post treatment with 1.38 μM salmon sperm DNA.

Figures 15A-C illustrate the secondary structure of hairpin H3 alone (15A), and the hybrids H3-T3 (15B) and H3-T3M1 (15C). H3 was derived from probe H1, described in Figure 5. Each of H3, T3, and T3M1 were ordered from Midland Certified Reagent Co. (Midland, Texas). The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figures 16A-C illustrate the effects of a single-base mismatch on the stability of the hybrids and the ability of the mismatch to maintain disruption of the hairpin formation (i.e., promoting fluorescence). CCD images illustrate the readily apparent differences in fluorescent intensity (compare Figures 16A-B). The graph presented in Figure 16C represents the binned CCD images, which reflect a nearly five-fold reduction in fluorescence intensity for the target possessing a single mismatch.

Figures 17A-C illustrate the hybridization-dependent fluorescence efficiency of a sensor chip containing hairpin probe HP1. Figure 17A-B are digital

images showing post-immobilization of hairpin probe HP1 (17A), and post treatment with 1.3 μM of hybridizing target sequence TP1 (17B). The graph presented in Figure 17C represents the binned CCD images, which illustrate a nearly 24-fold increase in fluorescence intensity upon target binding.

Figures 18A-C illustrate the hybridization-dependent fluorescence efficiency of a sensor chip containing hairpin probe HP2. Figure 18A-B are digital images showing post-immobilization of hairpin probe HP2 (18A), and post treatment with 2.6 μM of hybridizing target sequence TP2 (18B). The graph presented in Figure 18C represents the binned CCD images, which illustrate a nearly six-fold increase in fluorescence intensity upon target binding.

Figures 19A-D illustrate the CCD images obtain pre- and post-hybridization for two chips, AB-3 and AB-4, both of which were prepared with hairpin probe overlay using two distinct hairpins that target different nucleic acids and have different fluorescent signals. One probe, designated AH2-Rhodamine, contains the fluorophore rhodamine, which produces peak emissions at around 585 nm; whereas the other probe, designated BH2-Cy5, contains the fluorophore Cy5, which produces peak emissions at around 670 nm. Thus, fluorescent emissions by the two probes can be discriminated spectrally. Chip AB-3 was incubated with 3.0μM of hybridizing target AH2C (the complement to probe AH2), and chip AB-4 was incubated with 3.0μM of hybridizing target BH2C (the complement to probe BH2). Figure 19A is a CCD image of chip AB-3 with two probes before hybridization, and Figure 19B is a CCD image of the same chip after hybridization with AH2C. Figure 19C is a CCD image of chip AB-4 with two probes before hybridization, and Figure 19D is a CCD image of chip AB-4 with two probes after hybridization with BH2C.

Figures 20A-F are binned images and fluorescent spectra showing the results of exposing chips AB-3 and AB-4 to the hybridizing targets AH2C and BH2C. Figure 20A illustrates the fluorescence spectra of probe AH2-Rhodamine on chip AB-3 before and after hybridization with AH2C. Figure 20B illustrates the fluorescence spectra of probe BH2-Cy5 on chip AB-3 before and after hybridization with AH2C. Figure 20C illustrates the binning results of CCD images from Figures 19A-B. For chip AB-3, the increase of Rhodamine is higher than that of Cy5, so the fluorescence increase of the chip is mainly due to probe AH2-AH2C hybridization, rather than BH2-AH2C hybridization. The increase in fluorescence emission for chip AB-3 is

about 3.6-fold. Figure 20D illustrates the fluorescence spectra of probe AH2-Rhodamine on chip AB-4 before and after hybridization with BH2C. Figure 20E illustrates the fluorescence spectra of probe BH2-Cy5 on chip AB-4 before and after hybridization with BH2C. Figure 20F illustrates the binning results of CCD images from Figures 19C-D. For chip AB-4, the increase of Cy5 is higher than that of Rhodamine, so the fluorescence increase of the chip is mainly due to probe BH2-BH2C hybridization, rather than AH2-BH2C hybridization. The increase in fluorescence emission for chip AB-4 is about 1.6-fold.

Figure 21 illustrates the secondary structure of a hairpin (SEQ ID NO: 6) targeted to *Bacillus anthracis pag*. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figure 22 illustrates the secondary structure of a hairpin (SEQ ID NO: 9) targeted to *Bacillus cereus* isoleucyl-tRNA synthetase (ileS1) gene. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figure 23 illustrates the secondary structure of a hairpin (SEQ ID NO: 10) targeted to a portion of the *Staphylococcus aureus* genome. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

Figure 24 illustrates the secondary structure of a hairpin (SEQ ID NO: 11) targeted to a portion of the *Staphylococcus aureus* genome. The folding structure was predicted using the computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to a sensor chip that can be used to detect the presence of target nucleic acid molecules in a sample. As shown in Figure 1, the sensor chip **10** includes: a fluorescence quenching surface **12**; one or

more nucleic acid molecules **14** (i.e., as probes) each having first and second ends with the first end bound to the fluorescence quenching surface, a first region **16a**, and a second region **16b** complementary to the first region; and a first fluorophore **18** bound to the second end of the nucleic acid molecule **14**.

Suitable nucleic acid probes can be DNA, RNA, or PNA. The nucleic acid probes of the present invention can also possess one or more modified bases, one or more modified sugars, one or more modified backbones, or combinations thereof. The modified bases, sugars, or backbones can be used either to enhance the affinity of the probe to a target nucleic acid molecule or to allow for binding to the fluorescence quenching surface as described hereinafter. Exemplary forms of modified bases are known in the art and include, without limitation, alkylated bases, alkynylated bases, thiouridine, and G-clamp (Flanagan et al., *Proc. Natl. Acad. Sci. USA* 30:3513-3518 (1999), which is hereby incorporated by reference in its entirety). Exemplary forms of modified sugars are known in the art and include, without limitation, LNA, 2'-O-methyl, 2'-O-methoxyethyl, and 2'-fluoro (see, e.g., Freier and Attmann, *Nucl. Acids Res.* 25:4429-4443 (1997), which is hereby incorporated by reference in its entirety). Exemplary forms of modified backbones are known in the art and include, without limitation, phosphoramidates, thiophosphoramidates, and alkylphosphonates. Other modified bases, sugars, and/or backbones can, of course, be utilized.

With the first and second regions **16a,16b** of the nucleic acid probes **14** being complementary to one another, the nucleic acid probes have, under appropriate conditions, either (i) a hairpin conformation with the first and second regions hybridized together (shown on the left side of Figure 1) or (ii) a non-hairpin conformation (shown on the right side of Figure 1). The conditions under which the hairpin conformation exists is when the nucleic acid probe is maintained below its melting temperature (i.e., considering the length of the first and second regions, the GC content of those regions, and salt concentration), and typically when the target nucleic acid is not present. The conditions under which the non-hairpin conformation exists is either when the first nucleic acid is maintained above its melting temperature and/or when the probe is hybridized to its target nucleic acid (as shown in Figure 1).

The overall length of the nucleic acid probe is preferably between about 12 and about 60 nucleotides, more preferably between about 20 and about 50 nucleotides, most preferably between about 30 and about 40 nucleotides. It should be

appreciated, however, that longer or shorter nucleic acids can certainly be used. The first and second regions of the nucleic acid probes are preferably at least about 4 nucleotides in length, more preferably at least about 5 nucleotides in length or at least about 6 nucleotides in length. In the preferred embodiments described above, the first and second regions can be up to about 28 nucleotides in length, depending on the overall length of the nucleic acid probe and the size of a loop region present between the first and second regions. It is believed that a loop region of at least about 4 or 5 nucleotides is needed to allow the hairpin to form. The first and second regions can be perfectly matched (i.e., having 100 percent complementary sequences that form a perfect stem structure of the hairpin conformation) or less than perfectly matched (i.e., having non-complementary portions that form bulges within a non-perfect stem structure of the hairpin conformation). When the first and second regions are not perfectly matched the first and second regions can be the same length or they can be different in length, although they should still have at least 4 complementary nucleotides.

Nucleic acid probes of the present invention can have their entire length or any portion thereof targeted to hybridize to the target nucleic acid molecule, which can be RNA or DNA. Thus, the entire probe can hybridize to a target sequence of the target nucleic acid molecule or, alternatively, a portion thereof can hybridize to a target sequence of the target nucleic acid molecule. When less than the entire nucleic acid probe is intended to hybridize to the target nucleic acid molecule, the portion thereof that does hybridize (to the target nucleic acid molecule) should be at least about 50 percent, preferably at least about 60 or 70 percent, more preferably at least about 80 or 90 percent, and most preferably at least about 95 percent of the nucleic acid probe length. When only a portion of the nucleic acid probe is intended to hybridize to the target nucleic acid molecule, that portion can be part of the first region, part of the second region, or spanning both the first and second regions.

Referring again to Figure 1, while the probe remains in the hairpin conformation the fluorophore **18** bound to the second end of the nucleic acid probe is brought into sufficiently close proximity to the fluorescence quenching surface such that the surface substantially quenches fluorescent emissions by the fluorophore. In contrast, while the probe remains in the non-hairpin conformation, the fluorophore **18** bound to the second end of the nucleic acid probe is no longer constrained in

proximity to the fluorescence quenching surface **12**. As a result of its physical displacement away from the quenching surface, fluorescent emissions by the fluorophore **18** are substantially free of any quenching (i.e., emission from the sensor chip becomes detectable).

Selection of suitable nucleic acid molecules for use as probes can be achieved by (i) identifying an oligonucleotide that can hybridize to the target nucleic acid and then designing a nucleic acid probe that includes the oligonucleotide as a component part of the first and/or second region, and optionally as a component part of any loop region between the first and second regions; (ii) by identifying naturally occurring hairpin structures within the predicted folding structure of a target nucleic acid molecule, as described in co-pending U.S. Provisional Patent Application to Miller et al., entitled "Method of Identifying Hairpin DNA Probes By Partial Fold Analysis," filed concurrently with this application and expressly incorporated by reference in its entirety; or (iii) using a combination of the above procedures, modifying a portion of a naturally occurring hairpin structure, e.g., modifying one or more bases in the first or second region to increase the stability of the resulting probe or the stability of the probe-target interaction.

The fluorescence quenching surface **18** is capable of quenching or absorbing the fluorescent emissions of the fluorophore within the desired bandwidth. The fluorescence quenching surface can exist as either a solid substrate or a coating applied to another (i.e., inert or functional) substrate. When the fluorescent quenching surface is applied to a substrate, the fluorescent quenching surface can exist over substantially the entire substrate or, alternatively, in a plurality of discrete locations on the substrate. To obtain the latter construction, the fluorescent quenching surface can either be applied to the substrate in only a few locations or, after applying to substantially the entire substrate, the fluorescence quenching material can be etched or removed from the substrate in all but the desired, discrete locations.

By way of example, sputtering of atoms or ions through a mask, photolithographic liftoff techniques, and electron beam lithography can be used. All three of these techniques can be used to pattern surfaces with fluorescence quenching agents in selected patterns with length scales as small as about 50 nm (or any larger patterns). Alternatively, soft lithography can be used to pattern the fluorescence quenching agent on the 500 nm scale and larger; or, also using soft lithography, the

fluorescence quenching surface remains unmodified but the nucleic acid hairpins are applied thereto in a pattern using a spotter (i.e., spotting the buffer containing the hairpin) to make patterns on the 10 micron scale.

Preferred materials for formation of the fluorescence quenching surface include conductive metals or metal alloys, which offer the ability to completely or nearly completely quench the fluorescence emissions of the fluorophore, as well as semiconductor materials, either with or without n- or p-doping. Suitable conductive metals or metal alloys include, without limitation, gold, silver, platinum, copper, cobalt, iron, aluminum, iron-platinum, etc. Of these, gold, silver, and platinum are typically preferred. Suitable semiconductor materials include, without limitation, intrinsic or undoped silicon, p-doped silicon (e.g., $(CH_3)_2Zn$, $(C_2H_5)_2Zn$, $(C_2H_5)_2Be$, $(CH_3)_2Cd$, $(C_2H_5)_2Mg$, B, Al, Ga, or In dopants), n-doped silicon (e.g., H_2Se, H_2S, CH_3Sn, $(C_2H_5)_3S$, SiH_4, Si_2H_6, P, As, or Sb dopants), alloys of these materials with, for example, germanium in amounts of up to about 10% by weight, mixtures of these materials, and semiconductor materials based on Group III element nitrides. Other semiconductors known in the art can also be used.

The nucleic acid probe can be bound to the fluorescent quenching surface using known nucleic acid-binding chemistry or by physical means, such as through ionic, covalent or other forces well known in the art (see, e.g., Dattagupta et al., *Analytical Biochemistry* 177:85-89 (1989); Saiki et al., *Proc. Natl. Acad. Sci. USA* 86:6230-6234 (1989); Gravitt et al., *J. Clin. Micro.* 36:3020-3027 (1998), each of which is hereby incorporated by reference in its entirety). Either a terminal base or another base near the terminal base can be bound to the fluorescent quenching surface. For example, a terminal nucleotide base of the nucleic acid probe can be modified to contain a reactive group, such as (without limitation) carboxyl, amino, hydroxyl, thiol, or the like, thereby allowing for coupling of the nucleic acid probe to the surface.

The fluorophore can be any fluorophore capable of being bound to a nucleic acid molecule. Suitable fluorophores include, without limitation, fluorescent dyes, proteins, and semiconductor nanocrystal particles. The fluorophore used in the present invention is characterized by a fluorescent emission maxima that is detectable either visually or using optical detectors of the type known in the art. Fluorophores having fluorescent emission maxima in the visible spectrum are preferred.

Exemplary dyes include, without limitation, Cy2™, YO-PRO™-1, YOYO™-1, Calcein, FITC, FluorX™, Alexa™, Rhodamine 110, 5-FAM, Oregon Green™ 500, Oregon Green™ 488, RiboGreen™, Rhodamine Green™, Rhodamine 123, Magnesium Green™, Calcium Green™, TO-PRO™-1, TOTO®-1, JOE, BODIPY® 530/550, Dil, BODIPY® TMR, BODIPY® 558/568, BODIPY® 564/570, Cy3™, Alexa™ 546, TRITC, Magnesium Orange™, Phycoerythrin R&B, Rhodamine Phalloidin, Calcium Orange™, Pyronin Y, Rhodamine B, TAMRA, Rhodamine Red™, Cy3.5™, ROX, Calcium Crimson™, Alexa™ 594, Texas Red®, Nile Red, YO-PRO™-3, YOYO™-3, R-phycocyanin, C-Phycocyanin, TO-PRO™-3, TOTO®-3, DiD DilC(5), Cy5™, Thiadicarbocyanine, and Cy5.5™. Other dyes now known or hereafter developed can similarly be used as long as their excitation and emission characteristics are compatible with the light source and non-interfering with other fluorophores that may be present (i.e., not capable of participating in fluorescence resonant energy transfer or FRET).

Attachment of dyes to the opposite end of the nucleic acid probe can be carried using any of a variety of known techniques allowing, for example, either a terminal base or another base near the terminal base to be bound to the dye. For example, 3'-tetramethylrhodamine (TAMRA) may be attached using commercially available reagents, such as 3'-TAMRA-CPG, according to manufacturer's instructions (Glen Research, Sterling, Virginia). Other exemplary procedures are described in, e.g., Dubertret et al., *Nature Biotech.* 19:365-370 (2001); Wang et al., *J. Am. Chem. Soc.*, 125:3214-3215 (2003); *Bioconjugate Techniques*, Hermanson, ed. (Academic Press) (1996), each of which is hereby incorporated by reference in its entirety.

Exemplary proteins include, without limitation, both naturally occurring and modified (i.e., mutant) green fluorescent proteins (Prasher et al., *Gene* 111:229-233 (1992); PCT Application WO 95/07463, each of which is hereby incorporated by reference in its entirety) from various sources such as *Aequorea* and *Renilla*; both naturally occurring and modified blue fluorescent proteins (Karatani et al., *Photochem. Photobiol.* 55(2):293-299 (1992); Lee et al., *Methods Enzymol. (Biolumin. Chemilumin.)* 57:226-234 (1978); Gast et al., *Biochem. Biophys. Res. Commun.* 80(1):14-21 (1978), each of which is hereby incorporated by reference in its entirety) from various sources such as *Vibrio* and *Photobacterium*; and phycobiliproteins of the type derived from cyanobacteria and eukaryotic algae (Apt et

al., *J. Mol. Biol.* 238:79-96 (1995); Glazer, *Ann. Rev. Microbiol.* 36:173-198 (1982); Fairchild et al., *J. Biol. Chem.* 269:8686-8694 (1994); Pilot et al., *Proc. Natl. Acad. Sci. USA* 81:6983-6987 (1984); Lui et al., *Plant Physiol.* 103:293-294 (1993); Houmard et al., *J. Bacteriol.* 170:5512-5521 (1988), each of which is hereby incorporated by reference in its entirety), several of which are commercially available from ProZyme, Inc. (San Leandro, CA). Other fluorescent proteins now known or hereafter developed can similarly be used as long as their excitation and emission characteristics are compatible with the light source and non-interfering with other fluorophores that may be present.

Attachment of fluorescent proteins to the opposite end of the nucleic acid probe can be carried using any of a variety of known techniques, for example, either a terminal base or another base near the terminal base can be bound to the fluorescent protein. Procedures used for tether dyes to the nucleic acid can likewise be used to tether the fluorescent protein thereto. These procedures are generally described in, e.g., *Bioconjugate Techniques*, Hermanson, ed. (Academic Press) (1996), which is hereby incorporated by reference in its entirety.

Nanocrystal particles or semiconductor nanocrystals (also known as Quantum Dot™ particles), whose radii are smaller than the bulk exciton Bohr radius, constitute a class of materials intermediate between molecular and bulk forms of matter. Quantum confinement of both the electron and hole in all three dimensions leads to an increase in the effective band gap of the material with decreasing crystallite size. Consequently, both the optical absorption and emission of semiconductor nanocrystals shift to the blue (higher energies) as the size of the nanocrystals gets smaller.

The core of the nanocrystal particles is substantially monodisperse. By monodisperse, it is meant a colloidal system in which the suspended particles have substantially identical size and shape, i.e., deviating less than about 10% in rms diameter in the core, and preferably less than about 5% in the core.

Particles size can be between about 1 nm and about 1000 nm in diameter, preferably between about 2 nm and about 50 nm, more preferably about 5 nm to about 20 nm (such as about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 1 8, 19, or 20 nm).

When capped nanocrystal particles of the invention are illuminated with a primary light source, a secondary emission of light occurs of a frequency that corresponds to the band gap of the semiconductor material used in the nanocrystal particles. The band gap is a function of the size of the nanocrystal particle. As a result of the narrow size distribution of the capped nanocrystal particles, the illuminated nanocrystal particles emit light of a narrow spectral range resulting in high purity light. Spectral emissions in a narrow range of no greater than about 60 nm, preferably no greater than about 40 nm and most preferably no greater than about 30 nm at full width half max (FWHM) are observed. Spectral emissions in even narrower ranges are most preferred.

The nanocrystal particles are preferably passivated or capped either with organic or inorganic passivating agents to eliminate energy levels at the surface of the crystalline material that lie within the energetically forbidden gap of the bulk interior. These surface energy states act as traps for electrons and holes that would normally degrade the luminescence properties of the material. Such passivation produces an atomically abrupt increase in the chemical potential at the interface of the semiconductor and passivating layer (Alivisatos, *J. Phys. Chem.* 100:13226 (1996), which is hereby incorporated by reference in its entirety). As a result, higher quantum efficiencies can be achieved.

Exemplary capping agents include organic moieties such as tri-n-octyl phosphine (TOP) and tri-n-octyl phosphine oxide (TOPO) (Murray et al., *J. Am. Chem. Soc.* 115:8706 (1993); Kuno et al., *J. Phys. Chem.* 106(23):9869 (1997), each of which is hereby incorporated by reference in its entirety), as well as inorganic moieties such as CdS-capped CdSe and the inverse structure (Than et al., *J. Phys. Chem.* 100:8927 (1996), which is hereby incorporated by reference in its entirety), ZnS grown on CdS (Youn et al., *J. Phys. Chem.* 92:6320 (1988), which is hereby incorporated by reference in its entirety), ZnS on CdSe and the inverse structure (Kortan et al., *J. Am. Chem. Soc.* 112:1327 (1990); Hines et al., *J. Phys. Chem.* 100:468 (1996), each of which is hereby incorporated by reference in its entirety), ZnSe-capped CdSe nanocrystals (Danek et al., *Chem. Materials* 8:173 (1996), which is hereby incorporated by reference in its entirety), and SiO_2 on Si (Wilson et al., *Science* 262:1242 (1993), which is hereby incorporated by reference in its entirety).

In general, particles passivated with an inorganic coating are more robust than organically passivated particles and have greater tolerance to processing conditions used for their incorporation into devices. Particles that include a "core" of one or more first semiconductor materials can be surrounded by a "shell" of a second semiconductor material.

Thus, the nanocrystal particles as used in the present invention can be formed of one or more semiconducting materials. Suitable semiconducting materials include, without limitation, a group IV material alone (e.g., Si and Ge), a combination of a group IV material and a group VI material, a combination of a group III material and a group V material, or a group II material and a group VI material. When a combination of materials are used, the semiconducting materials are presented in a "core/shell" arrangement.

Suitable core/shell material combinations include, without limitation, group IV material forming the core and group VI materials forming the shell; group III material forming the core and group V materials forming the shell; and group II material forming the core and group VI materials forming the shell. Exemplary core/shell combinations for groups IV/VI are: Pb and one or more of S, Se, and Te. Exemplary core/shell combinations for groups III/V are: one or more of Ga, In, and Al as the group III material and one or more of N, P, As, and Sb as the group V material. Exemplary core/shell combinations for groups II/VI are: one or more of Cd, Zn, and Hg as the group II material, and one or more of S, Se, and Te as the group VI material. Other combinations now known or hereinafter developed can also be used in the present invention.

Fluorescent emissions of the resulting nanocrystal particles can be controlled based on the selection of materials and controlling the size distribution of the particles. For example, ZnSe and ZnS particles exhibit fluorescent emission in the blue or ultraviolet range (~400 nm or less); Au, Ag, CdSe, CdS, and CdTe exhibit fluorescent emission in the visible spectrum (between about 440 and about 700 nm); InAs and GaAs exhibit fluorescent emission in the near infrared range (~1000 nm), and PbS, PbSe, and PbTe exhibit fluorescent emission in the near infrared range (i.e., between about 700-2500 nm). By controlling growth of the nanocrystal particles it is possible to produce particles that will fluoresce at desired wavelengths. As noted

above, smaller particles will afford a shift to the blue (higher energies) as compared to larger particles of the same material(s).

Preparation of the nanocrystal particles can be carried out according to known procedures, e.g., Murray et al., *MRS Bulletin* 26(12):985-991 (2001); Murray et al., *IBM J. Res. Dev.* 45(1):47-56 (2001); Sun et al., *J. Appl. Phys.* 85(8, Pt. 2A): 4325-4330 (1999); Peng et al., *J. Am. Chem. Soc.* 124(13):3343-3353 (2002); Peng et al., *J. Am. Chem. Soc.* 124(9):2049-2055 (2002); Qu et al., *Nano Lett.* 1(6):333-337 (2001); Peng et al., *Nature* 404(6773):59-61 (2000); Talapin et al., *J. Am. Chem. Soc.* 124(20):5782-5790 (2002); Shevenko et al., *Advanced Materials* 14(4):287-290 (2002); Talapin et al., *Colloids and Surfaces, A: Physiochemical and Engineering Aspects* 202(2-3):145-154 (2002); Talapin et al., *Nano Lett.* 1(4):207-211 (2001), each of which is hereby incorporated by reference in its entirety.

Whether in a core/shell arrangement or otherwise passivated with other compounds, the nanocrystal particles can also be rendered water soluble, if so desired. To make water-soluble nanocrystal particles, hydrophilic capping compounds are bound to the particles. One suitable class includes carboxylic acid capping compounds with a thiol functional group (forming a sulfide bridge with the nanocrystal particle), which can be reacted with the nanocrystal. Exemplary capping compounds include, without limitation, mercaptocarboxylic acid, mercaptofunctionalized amines (e.g., aminoethanethiol-HCl, homocysteine, or 1-amino-2-methyl-2-propanethiol-HCl), mercaptofunctionalized sulfonates, mercaptofunctionalized alkoxides, mercaptofunctionalized phosphates and phosphonates, aminofunctionalized sulfonates, aminofunctionalized alkoxides, aminofunctionalized phosphates and phosphonates, phosphine(oxide)functionalized sulfonates, phosphine(oxide)functionalized alkoxides, phosphine(oxide)functionalized phosphates and phosphonates, and combinations thereof. Procedures for binding these capping compounds to the nanocrystal particles are known in the art, e.g., U.S. Patent No. 6,319,426 to Bawendi et al., which is hereby incorporated by reference in its entirety.

Attachment of a nanocrystal particle to the opposite end of the nucleic acid probe can be carried using any of a variety of known techniques, for example, either a terminal base or another base near the terminal base can be bound to the nanocrystal particle. Procedure used for tether dyes to the nucleic acid can likewise be

used to tether the nanocrystal particle thereto. Details on these procedures are described in, e.g., *Bioconjugate Techniques*, Hermanson, ed. (Academic Press) (1996), which is hereby incorporated by reference in its entirety.

Having identified the sequence of a nucleic acid molecule to be used as a probe in a sensor of the present invention, and having selected the appropriate fluorophore and fluorescence quenching surface to be utilized, the sensor of the present invention can be assembled using the above-described procedures. Attachment of the fluorophore to one end of the nucleic acid probe can be carried out prior to attachment of the opposite end of the nucleic acid probe to the fluorescence quenching surface, or *vice versa*. Alternatively, the probe can be ordered from any one of various vendors that specialize in preparing oligonucleotides to desired specifications (i.e., having one end modified for binding to the fluorescence quenching surface and the other end bound by a fluorophore) and thereafter attached to the fluorescence quenching surface. Two exemplary vendors are Midland Certified Reagent Co. (Midland, Texas) and Integrated DNA Technologies, Inc. (Coralville, Iowa).

In preparing the sensor chips of the present invention, a competitor (or spacer) molecule can also be attached to the fluorescence quenching surface, either as a separate step or as a single step (i.e., using a solution containing both the nucleic acid probe and the competitor molecule). The role of the competitor molecule is simply to minimize the concentration (and promote dispersion) of nucleic acid probes bound to the fluorescence quenching surface, thereby inhibiting the likelihood of interference between adjacent nucleic acid probes, which could result in background fluorescence. Like the nucleic acid probes, the competitor molecule contains a reactive group such as (without limitation) carboxyl, amino, hydroxyl, thiol, or the like, thereby allowing for coupling of the competitor molecule to the fluorescence quenching surface. Preferred competitor molecules include, without limitation, thiol-containing compounds, such as mercaptopropanol, cysteine, thiooctic acid, 2-mercaptoethanol, 3-mercapto-2-butanol, 2-mercapto,1,2-propanediol, 2-(butylamino)ethanethiol, 2-dimethylaminoethanethiol, 2-diethylaminoethanethiol, 3-mercaptopropionic acid, etc.

According to one approach, the fluorescence quenching surface is first exposed to a solution containing the competitor molecule and allowed to self-

assemble (to the surface) for a sufficient length of time. Thereafter, the modified surface is secondly exposed to a solution containing the nucleic acid probe and allowed to self-assemble (to the surface) for a sufficient length of time. As is well known in the art, the exposure time to one or both of the solutions can vary according to the concentrations of the competitor molecule and the nucleic acid probe in their respective solutions. After each exposure, the fluorescence quenching surface can be rinsed with pure water or saline solution, preferably at elevated temperatures so as to remove unbound competitor or unbound nucleic acid probe, respectively.

According to another approach, the fluorescence quenching surface is simultaneously exposed to a solution containing both the competitor molecule and the nucleic acid probe, and allowed to self-assemble for a sufficient length of time. As noted above, the exposure time to the combined solution can vary according to the concentrations of the competitor molecule and the nucleic acid probe. After exposure, the fluorescence quenching surface can be briefly rinsed with pure water or saline solution, preferably at elevated temperatures so as to remove unbound competitor and/or unbound nucleic acid probe. The resulting sensor chip can then be used to detect the presence of target nucleic acid molecules in sample preparations.

The ratio of the competitor molecule to the nucleic acid probe is preferably between about 2:1 and about 18:1, more preferably between about 5:1 and about 15:1, most preferably between about 8:1 and about 12:1.

The sensor chip can have a number of configurations depending on the nature and number of target nucleic acid molecules to be identified by a single chip.

According to one embodiment, the sensor chip is constructed using one or more nucleic acid probes, whether the same or different, all of which are directed to the same target molecule (perhaps, however, at different locations on the target). In this case, the probes can be attached to the fluorescence quenching surface in any location or over the substantially entire surface thereof.

According to another embodiment, shown in Figure 2, the sensor chip **10a** is constructed using two or more nucleic acid probes **14,14'** each having a different target nucleic acid molecule, where each of the two or more nucleic acid probes **14,14'** is localized to a specific region **A,B** on the fluorescence quenching surface **12**. One probe **14** (and its target) can be distinguished from another probe **14'** (and its target) by the localization of any fluorescence emissions from the sensor chip

10a. In this arrangement, the fluorophores **18** used on the two or more nucleic acid probes **14,14'** can be the same or they can be different.

According to another embodiment, shown in Figure 3, the sensor chip **10b** is constructed using two or more nucleic acid probes **14,14'** each having a different target nucleic acid molecule, where the two or more nucleic acid probes are co-localized (i.e., overlapping locations) over the fluorescence quenching surface **12** or portions thereof. In this arrangement, the fluorophores **18,18'** used on the two or more nucleic acid probes are different so that fluorescent emissions from each can be distinguished from any others.

To distinguish between multiple fluorescent emissions emanating from a single location on the surface of the sensor chip (i.e., signal from one probe rather than another), the fluorescent emissions need only differ sufficiently to allow for resolution by the detector being utilized. Resolution of the signals can also depend, in part, on the nature of the emission pattern. For example, narrow emission maxima are more easily resolved than broad emission maxima that may interfere with emissions by other fluorophores. Thus, the selection of fluorophores should be made so as to minimize the interference given the sensitivity of the detector being utilized. By way of example, highly sensitive detectors can discriminate between the narrow emission maxima of semiconductor nanocrystals and dyes, allowing for separation of emission maxima that differ by about 1 nm or greater. Preferably, however, the emission maxima between the two or more fluorophores will differ by about 10 nm or greater or even 20 nm or greater, more preferably 30 nm or greater or even 40 nm or greater. Generally, the greater the separation between the emission maxima of the two or more fluorophores, the easier it will be to resolve their signals from overlapping locations on the surface of the sensor chip.

The sensor chip is intended to be used as a component in a biological sensor device or system. Basically, the device includes, in addition to the sensor chip, a light source that illuminates the sensor chip at a wavelength suitable to induce fluorescent emissions by the fluorophores associated with the one or more probes bound to the chip, and a detector positioned to capture any fluorescent emissions by the fluorophores.

The light source can be any light source that is capable of inducing fluorescent emissions by the selected fluorophores. Light sources that provide

illumination wavelengths between about 200 nm and about 2000 nm are preferred. Exemplary light sources include, without limitation, lasers and arc lamps. Typical powers for lasers are at least about 1 mW; however, when used with an objective lens focusing the laser light to a small spot, as little as about 1 μW is sufficient. By way of example, Xenon arc lamps should be at least about 75 W.

The detector can be any detection device that is capable of receiving fluorescent emissions and generating a response to be examined by an operator of the biological sensor device. Suitable detectors include, without limitation, charge coupled devices (CCDs), photomultiplier tubes (PMTs), avalanche photodiodes (APDs), and photodiodes that contain a semiconductor material such as Si, InGaAs, extended InGaAs, Ge, HgCdTe, PbS, PbSe, or GaAs to convert optical photons into electrical current. Of these suitable detectors, the CCD is preferred because it can produce an image in extremely dim light, and its resolution (i.e., sharpness or data density) does not degrade in low light.

In addition to the above components, the biological sensor device can also include a notch filter positioned between the light source and the sensor chip and/or a bandpass filter positioned between the sensor chip and the detector. The notch filter will screen out a narrow band of photoradiation, i.e., at or near the excitation maxima of the selected fluorophore(s), so as to minimize any background excitation by materials present in or on the sensor chip or by non-quenched fluorophore(s). The bandpass filters control the spectral composition of transmitted energy, typically though not exclusively by the effects of interference, resulting in high transmission over narrow spectral bands. By way of example, the bandpass filter can allow passage of light within a range that is not more than about 10 nm greater or less than the wavelength of the maximum emissions of the fluorophore(s). When two or more fluorophores are used having different emission maxima, the bandpass filter will emit passage of light within a larger wavelength band that extends from slightly below than the lowest wavelength maxima up to slightly higher than the highest wavelength maxima. Alternatively, when multiple fluorophores are used the emission signal can be split prior to passage through any filters (i.e., one for each fluorophore). Each split emission signal can include a separate bandpass filter that is configured for the emission maxima of one fluorophore but not the others.

By way of example, Figure 4 shows the configuration of one particular embodiment of the biological sensor device **50**. The device includes a light source **52** that produces a focused beam of light **L** which is directed through a notch filter **54** and through an inverted microscope **56** (as shown, the notch filter is a component of the inverted microscope), where it contacts the sensor chip **10** placed on a sample stage. Any fluorescent emissions are captured by the inverted microscope **56** and the signal passes through a bandpass filter **58** prior to reaching the detector device **60**. As shown, the detector device **60** includes a spectrophotometer **62** coupled to a CCD **64**, whose electrical output signal is directed to a personal computer **66** or similar device capable of receiving the electrical output and generating an image of the detected fluorescence emitted from the sensor chip **10**.

The sample is preferably present in the form of a buffered solution or other medium suitable for use during hybridization. The sample itself can be either a clinical or environmental sample to which buffer or buffer salts are added, derived from purification of DNA or RNA from clinical or environmental specimens, or the product of a PCR reaction, etc. Basically, the sample can be in any form where the suspected nucleic acid target is maintained in a substantially stable manner (i.e., without significant degradation).

During use of the biological sensor device and the associated sensor chip, the presence of a target nucleic acid molecule in a sample can be achieved by first exposing the sensor chip to a sample under conditions effective to allow any target nucleic acid molecule in the sample to hybridize to the first and/or second regions of the nucleic acid probe(s) present on the sensor chip, illuminating the sensor chip with light sufficient to cause emission of fluorescence by the fluorophore(s), i.e., associated with the nucleic acid probe(s), and then determining whether or not the sensor chip emit(s) detectable fluorescent emission (of the fluorophore(s)) upon said illuminating. When fluorescent emission by the fluorophore(s) is detected from the chip, that indicates that the nucleic acid probe is in the non-hairpin conformation and therefore that the target nucleic acid molecule is present in the sample.

The conditions utilized during the exposure step include hybridization and then wash conditions, as is typical during hybridization procedures. The hybridization and wash conditions can be carried in buffered saline solutions either at or slightly above room temperature (i.e., up to about 30°C). Alternatively, as is known

in the art, the hybridization conditions can be selected so that stringency will vary. That is, lower stringency conditions will discriminate less between perfectly matched target nucleic acid molecules and non-perfectly matched nucleic acid molecules, whereas higher stringency conditions will discriminate between perfectly matched and non-perfectly matched nucleic acid molecules. In general, the highest stringency that can be tolerated by the probe and the intended target can be selected so as to minimize or completely avoid the possibility of a false positive response caused by hybridization to non-perfectly matched nucleic acid molecules. Alternatively, it may be desirable to begin hybridization at a temperature above the melting temperature of the hairpin probe, thus promoting an open conformation, and then during the course of the hybridization procedure allowing the chip to cool so that hairpins not participating in hybridization (i.e., in cases where there is no complementarity) to re-fold, and fluorescence to be quenched. The latter procedure would be desirable, for example, when the hairpin probe is quite stable (having a predicted E value in the range of about -9 to about -12 kcal/mol), even in the presence of target nucleic acid molecules. In either case though, detection typically is not carried out until the hybridization and wash procedures have been completed.

An example of suitable stringency conditions is when hybridization is carried out at a temperature of at least about 35°C using a hybridization medium that includes about 0.3M Na^+, followed by washing at a temperature of at least about 35°C with a buffer that includes about 0.3M Na^+ or less. Higher stringency can readily be attained by increasing the temperature for either hybridization or washing conditions or decreasing the sodium concentration of the hybridization or wash medium. Other factors that affect the melting temperature of the hairpin probe include its GC content and the length of the stem (and whether the stem perfectly hybridizes intramolecularly). Nonspecific binding may also be controlled using any one of a number of known techniques such as, for example, addition of heterologous RNA, DNA, and SDS to the hybridization buffer, treatment with RNase, etc. Wash conditions can be performed at or below stringency of the hybridization procedure, or even at higher stringency when so desired. Exemplary high stringency conditions include carrying out hybridization at a temperature of about 50°C to about 65°C (from about 1 hour up to about 12 hours) in a hybridization medium containing 2X SSC buffer (or its equivalent), followed by washing carried out at between about 50°C to

about 65°C in a 0.1X SSC buffer (or its equivalent). Variations on the hybridization conditions can be carried out as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, NY (1989), which is hereby incorporated by reference in its entirety.

The nucleic acid probes, used in preparing sensor chips of the present invention, can be selected so that they hybridize to target nucleic acid molecules that are specific to pathogens, are associated with disease states or conditions, contain polymorphisms that may or may not be associated with a disease state but can also be a forensic target or associated with a breeding trait for plants or animal. Other uses should be appreciated by those of ordinary skill in the art.

By way of example, a number of specific nucleic acid probes have been identified. The nucleotide sequences and their targets are identified below:

SEQ ID NO: 1 (targeted to *Staphylococcus aureus FemA*) has the nucleotide sequence
acacgctcatcataaccttcagcaagctttaactcatagtgagcgtgt
and is characterized by the putative folding structure illustrated in Figure 5.

SEQ ID NO: 2 (targeted to *Staphylococcus aureus mecR*) has the nucleotide sequence
aatgatgataacaccttctacacctccataatcatcatt
and is characterized by the putative folding structure illustrated in Figure 6.

SEQ ID NO: 3 (targeted to *Exophiala dermatitidis* 18S ribosomal RNA gene) has the nucleotide sequence
ggtctggtcgagcgtttccgcgcgaccctcccaaagaca
and is characterized by the putative folding structure illustrated in Figure 9.

SEQ ID NO: 4 (targeted to *Trichophyton tonsurans* strain 18S ribosomal RNA gene) has the nucleotide sequence
gttcggcgagcctctctttatagcggctcaacgctggac
and is characterized by the putative folding structure illustrated in Figure 10.

SEQ ID NO: 5 (targeted to *Bacillus anthracis pag*) has the nucleotide sequence
tcgttagtgttaggaaaaaatcaaacactcgcga
and is characterized by the putative folding structure illustrated in Figures 8A.

SEQ ID NO: 6 (targeted to *Bacillus anthracis pag*) has the nucleotide sequence
tttctttcaccatggatttctaatattcatgaaaagaaa
and is characterized by the putative folding structure illustrated in Figure 21.

SEQ ID NO: 7 (targeted to *Bacillus anthracis pag*) has the nucleotide sequence
tcttcaccatggatttctaatatccatgaaaaga
and is characterized by the putative folding structure illustrated in Figure 7A.

SEQ ID NO: 8 (targeted to *Bacillus cereus* isoleucyl-tRNA synthetase (ileS1) gene) has the nucleotide sequence
cgtgattcattagttatgctaggagatcacg
and is characterized by the putative folding structure illustrated in Figure 22.

SEQ ID NO: 9 (targeted to a portion of *Staphylococcus aureus* complete genome located between ORFID:SA0529 and ORFID:SA0530) has the nucleotide sequence
cgataatatgatgcctaggcagaaatattatcg
and is characterized by the putative folding structure illustrated in Figure 23.

SEQ ID NO: 10 (targeted to a portion of *Staphylococcus aureus* complete genome located between ORFID:SA0529 and ORFID:SA0530 and including several bases within the latter open reading frame) has the nucleotide sequence
tatcaataataaacgaataggggtgttaatattgata
and is characterized by the putative folding structure illustrated in Figure 24.

Pathogens that can be identified using the products and processes of the present invention include any bacteria, fungi, viruses, rickettsiae, chlamydiae, and parasites, but preferably those identified as belonging within the classifications listed as Biosafety Levels Two, Three, and Four by the U.S. Centers for Disease Control and Prevention, the National Institutes of Health, and the World Health Organization.

Exemplary bacterial pathogen that can be identified in accordance with the present invention include, without limitation: *Acinetobacter calcoaceticus*, *Actinobacillus* species (all species), *Aeromonas hydrophila*, *Amycolata autotrophica*, *Arizona hinshawii* (all serotypes), *Bacillus anthracis*, *Bartonella* species (all species), *Brucella* species (all species), *Bordetella* species (all species), *Borrelia* species (e.g.,

B. recurrentis, *B. vincenti*), *Campylobacter* species (e.g., *C. fetus*, *C. jejuni*), *Chlamydia* species (e.g., *Chl. psittaci*, *Chl. trachomatis*), *Clostridium* species (e.g., *Cl. botulinum*, *Cl. chauvoei*, *Cl. haemolyticum*, *Cl. histolyticum*, *Cl. novyi*, *Cl. septicum*, *Cl. tetani*), *Corynebacterium* species (e.g., *C. diphtheriae*, *C. equi*, *C. haemolyticum*, *C. pseudotuberculosis*, *C. pyogenes*, *C. renale*), *Dermatophilus congolensis*, *Edwardsiella tarda*, *Erysipelothrix insidiosa*, *Escherichia coli* (e.g., all enteropathogenic, enterotoxigenic, enteroinvasive and strains bearing K1 antigen), *Francisella tularensis*, *Haemophilus* species (e.g., *H. ducreyi*, *H. influenzae*), *Klebsiella* species (all species), *Legionella pneumophila*, *Leptospira interrogans* (e.g., all serotypes), *Listeria* species (all species), *Moraxella* species (all species), *Mycobacteria* species (all species), *Mycobacterium avium*, *Mycoplasma* species (all species), *Neisseria* species (e.g., *N. gonorrhoea*, *N. meningitides*), *Nocardia* species (e.g., *N. asteroides*, *N. brasiliensis*, *N. otitidiscaviarum*, *N. transvalensis*), *Pasteurella* species (all species), Pseudomonas species (e.g., *Ps. mallei*, *Ps. pseudomallei*), *Rhodococcus equi*, *Salmonella* species (all species), *Shigella* species (all species), *Sphaerophorus necrophorus*, *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus* species (e.g., *S. pneumoniae*, *S. pyogenes*), *Treponema* species (e.g., *T. carateum*, *T. pallidum*, and *T. pertenue*), *Vibrio* species (e.g., *V. cholerae*, *V. parahemolyticus*), and *Yersinia* species (e.g., *Y. enterocolitica*, *Y. pestis*).

Exemplary fungal pathogens that can be identified in accordance with the present invention include, without limitation: *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Paracoccidioides braziliensis*, *Trypanosoma cruzi*, *Coccidioides immitis*, *Pneumocystis carinii*, and *Histoplasma* species (e.g., *H. capsulatum*, *H. capsulatum* var. *duboisii*).

Exemplary parasitic pathogens that can be identified in accordance with the present invention include, without limitation: *Endamoeba histolytica*, *Leishmania* species (all species), *Naegleria gruberi*, *Schistosoma mansoni*, *Toxocara canis*, *Toxoplasma gondii*, *Trichinella spiralis*, and *Trypanosoma cruzi*.

Exemplary viral, rickettsial, and chlamydial pathogens that can be identified in accordance with the present invention include, without limitation: Adenoviruses (all types), Cache Valley virus, Coronaviruses, Coxsackie A and B viruses, Cytomegaloviruses, Echoviruses (all types), Encephalomyocarditis virus (EMC), Flanders virus, Hart Park virus, Hepatitis viruses-associated antigen material,

Herpesviruses (all types), Influenza viruses (all types), Langat virus, Lymphogranuloma venereum agent, Measles virus, Mumps virus, Parainfluenza virus (all types), Polioviruses (all types), Poxviruses (all types), Rabies virus (all strains), Reoviruses (all types), Respiratory syncytial virus, Rhinoviruses (all types), Rubella virus, Simian viruses (all types), Sindbis virus, Tensaw virus, Turlock virus, Vaccinia virus, Varicella virus, Vesicular stomatitis virus, Vole rickettsia, Yellow fever virus, Avian leukosis virus, Bovine leukemia virus, Bovine papilloma virus, Chick-embryo-lethal orphan (CELO) virus or fowl adenovirus 1, Dog sarcoma virus, Guinea pig herpes virus, Lucke (Frog) virus, Hamster leukemia virus, Marek's disease virus, Mason-Pfizer monkey virus, Mouse mammary tumor virus, Murine leukemia virus, Murine sarcoma virus, Polyoma virus, Rat leukemia virus, Rous sarcoma virus, Shope fibroma virus, Shope papilloma virus, Simian virus 40 (SV-40), Epstein-Barr virus (EBV), Feline leukemia virus (FeLV), Feline sarcoma virus (FeSV), Gibbon leukemia virus (GaLV), Herpesvirus (HV) ateles, Herpesvirus (HV) saimiri, Simian sarcoma virus (SSV)-1, Yaba, Monkey pox virus, Arboviruses (all strains), Dengue virus, Lymphocytic choriomeningitis virus (LCM), Rickettsia (all species), Yellow fever virus, Ebola fever virus, Hemorrhagic fever agents (e.g., Crimean hemorrhagic fever, (Congo), Junin, and Machupo viruses, Herpesvirus simiae (Monkey B virus), Lassa virus, Marburg virus, Tick-borne encephalitis virus complex (e.g., Russian spring-summer encephalitis, Kyasanur forest disease, Omsk hemorrhagic fever, and Central European encephalitis viruses), and Venezuelan equine encephalitis virus.

Thus, a further aspect of the present invention relates to a method of detecting presence of a pathogen in a sample that is carried out by performing the above-described method (of detecting the presence of the target nucleic acid molecule) when using a sensor chip having a nucleic acid probe with at least portions of the first and/or second region thereof specific for hybridization with a target nucleic acid molecule of a pathogen.

Yet another aspect of the present invention relates to a method of genetic screening that is carried out by performing the above-described method (of detecting the presence of the target nucleic acid molecule) when using a sensor chip having a nucleic acid probe with at least portions of the first and/or second region thereof specific for hybridization with a genetic marker. As noted above, the genetic marker can be associated with disease states or conditions, contain polymorphisms

that may or may not be associated with a disease state but can also be a forensic target or associated with a breeding trait for plants or animal

EXAMPLES

The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1 - Predicted Secondary Structure of H1 and H2

Two DNA hairpins, H1 and H2 (Table 1 below), were designed to incorporate portions of the *Staphylococcus aureus FemA* (Berger-Bachi et al., *Mol. Gen. Genet.* 219:263-269 (1989); Genbank accession X17688, each of which is hereby incorporated by reference in its entirety) and *mecR* (Archer et al., *Antimicrob. Agents. Chemother.* 38:447-454 (1994), which is hereby incorporated by reference in its entirety) methicillin-resistance genes, and bearing a 5' end-linked thiol and a 3' end-linked rhodamine. After designing the nucleic acid molecules H1 and H2, they were ordered from Midland Certified Reagent Co. (GF-grade), and used as supplied.

Table 1: Sequences used in Examples 1-4

Entry	SEQ ID NO:	Sequence
H1	1	(C6Thiol)-acacgctcatcataaccttcagcaagctttaactcatagtgagcgtgt-Rhodamine
T1	11	acgctcactatgagttaaagcttgctgaaggttatga
H2	2	(C6Thiol)-aatgatgataacaccttctacacctccataatcatcatt- Rhodamine
T2	12	tatggaggtgtagaaggtgttatcatcatt

Both H1 and H2, and their respective complementary strands T1 and T2, were obtained from a commercial supplier.

The computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety) was used to predict the secondary structures of H1 and H2 prior to synthesis. Predicted lowest energy structures (using parameters derived from Santa Lucia, Jr., *Proc. Natl. Acad. Sci. USA* 95:1460-1465 (1998), which is hereby incorporated by reference in its entirety) are shown in Figures 5 and 6, respectively. These computational predictions of the hairpin secondary structure for H1 and H2 were confirmed through thermal melting experiments.

All thermal melts were conducted on a Gilford spectrophotometer, with the oligonucleotide dissolved in 0.5 M NaCl Buffer (20 mM cacodylic acid and 0.5 mM EDTA, 0.5 M NaCl, pH = 7; all H_2O used in the preparation of buffers was 18.2 MΩ, as produced by a Barnstead Nanopure system). Each sample was warmed to 80°C, then cooled back to 10°C prior to running the melting experiment. Results are shown in Figures 11A-D. Measured melting temperatures of H1 and H2 were 69°C and 58°C, respectively.

Example 2 - Preparation of Substrate and DNA Immobilization

Glass slides were cleaned with piranha etch solution (4:1 concentrated H_2SO_4/30% H_2O_2) overnight at room temperature, and then rinsed with ultrapure water. Metal deposition was performed at a rate of 0.2 nm/s using Denton Vacuum Evaporator (DV-502A). First, a chromium adhesion layer of 7 nm was coated on the glass, followed by 100 nm thick gold film. Before use, the gold substrates were annealed at 200°C for one hour and cleaned with piranha solution for 0.5 hr.

The gold-coated substrate was soaked in a mixture solution of hairpin oligonucleotide and 3-mercapto-1-propanol (MP) (Aldrich Chemical Company, used without further purification) at a ratio of 1 to 10 for self-assembly. Two hours later, the substrate was thoroughly rinsed with hot water (90°C or higher; H_2O used in the rinse solution was 18.2 MΩ, as produced by a Barnstead Nanopure system) to remove unbound DNA. Next, the substrate carrying the mixed monolayer was immersed in 0.5 M NaCl buffer (20 mM Cacodylic acid, 0.5 mM EDTA, 0.5 M NaCl, pH = 7). Optimization of both the DNA to MP ratio and the immersion time were useful in obtaining the most efficient increase in fluorescence intensity. Longer incubation times and lower relative concentrations of MP would be expected to result in Au surfaces with larger amounts of bound DNA. However, these conditions should also lead to complications resulting from nonspecific adhesion of DNA to the surface (Gearhart et al., *J. Phys. Chem. B* 105:12609-12615 (2001), which is hereby incorporated by reference in its entirety), or by a lack of sufficient interstitial space for high hybridization efficiency (Lin et al., *J. Langmuir* 18:788-796 (2002), which is hereby incorporated by reference in its entirety). Indeed, as described in greater detail

below, it was found that significant deviation from the conditions described above can result in significant background fluorescence intensity.

A number of different MP:probe solutions were prepared and allowed to self-assemble to gold-coated substrate. Thereafter, the resulting sensor chips were examined for their fluorescence efficiency (i.e., comparing pre- and post-hybridization fluorescence).

Table 2: Relationship of MP:Probe Concentration Ratio and Chip Performance

Probe conc. (μM)	MP:Probe Ratio	Fold-Increase in Chip Fluorescence	Comment
0.13	0:1	0.73	Background signal is 4 times higher than with MP
0.13	1:1	1.53	Low FL intensity
0.13	5:1	5.13	Good FL intensity
0.13	10:1	23.7	Near optimal FL intensity
0.13	20:1	1.95	Low FL intensity
0.13	30:1	1.87	Low FL intensity

To achieve a higher fold-increase in fluorescence for post-hybridization relative to pre-hybridization, there should be a lower background signal and, thus, better quenching efficiency. In addition, high hybridization efficiency is desirable, which means that most hairpins on the surface form a duplex in the presence of the target. MP was used as a competitor (i.e., spacer) molecule for binding to the surface of the chip. In the absence of such a competitor (i.e, 0:1 ratio), the probes were densely packed on the chip surface and, as a result, there was not enough interstitial space for them to form hairpin configuration and non-specific binding could not be avoided. Consequently, quenching of fluorophores by gold is poor, resulting in higher background signal. In contrast, when the competitor is present in too high a ratio (i.e., 20:1 or greater), the competitor will have superiority to bind the chip surface. As a result, this leads to not enough probes and lower signal during post-hybridization detection. Optimal MP:probe ratio provides enough space for duplex formation during hybridization and results in improved performance of the chip.

Example 3 - Construction of Fluorescent Detection System Using H1- and H2-Functionalized Gold

Fluorescence measurement was performed on a Nikon inverted fluorescence microscope equipped with a liquid nitrogen cooled charge coupled

device (CCD) (Figure 4). An Ar^+ laser was used for excitation at 514 nm. The beam passed through a high-pass dichroic mirror and a notch filter before it reached the sample. The DNA chip was inverted on a clean cover slip on top of a 60× air objective. Fluorescence emission was collected by the same objective and directed through a bandpass filter (585 nm ± 5 nm, ensuring only fluorescence from rhodamine was observed) to a CCD. In order to track the fluorescence of a certain area, a pattern was scratched on the gold so that exactly the same area could be examined before and after hybridization for comparison. At least four areas at different positions of the gold were chosen for each sample during fluorescence measurement. Under laser illumination, the images were recorded by the CCD camera at 10s integration time.

Example 4 - Hybridization of Targets T1 and T2 to H1- and H2-Functionalized Gold Films

Having prepared the sensor chip containing hairpin probes H1 and H2 bound to the gold surface, hybridization of their respective targets, T1 and T2, was performed at room temperature for 16 hours under the same buffer conditions (e.g., in 0.5 M NaCl buffer containing 20 mM Cacodylic acid, 0.5 mM EDTA, 0.5 M NaCl, pH = 7). The 16-hour incubation time was chosen primarily for convenience; preliminary experiments with the hybridization of T1 to H1 suggest that shorter incubation times are possible (see Table 3 below).

Table 3: Affect of Hybridization Time on Fluorescent Detection

Hybridization Time (in minutes):	10	20	30	60	120	240	360
Fluorescence increase (fold)	4.1	5.8	6.1	9.0	12.2	11.1	10.5

Using epi-fluorescence confocal microscopy, the fluorescence of H1 and H2-functionalized gold films was examined in the presence and absence of T1 and T2, respectively (compare Figures 12A and 13A with Figures 12B and 13B). As noted above in Example 3, films were excited at 514 nm. Strong reflected laser scatter was removed using the dichroic beamsplitter and a laser-line notch filter. Sample emission was collected by a CCD attached to an imaging spectrograph and passed through a band-pass filter (585nm ± 5nm) to ensure that only rhodamine fluorescence was being observed.

Fluorescence quenching of the hairpins prior to addition of T1 or T2 was calculated as follows:

$$Q = 100 \times \{1 - (I_{probe} - I_{blank}) / (I_{target} - I_{blank})\}$$

where:

I_{probe} The fluorescence intensity of hairpin probe on gold before hybridization

I_{target} The fluorescence intensity of hairpin probe on gold after hybridization with the target sequences

I_{blank} The fluorescence intensity of background including bare gold, cover slip and buffer.

The fluorescence quenching by the gold surface was found to be 96 ± 3% for H1 (Figure 13A) and 95± 4% for H2 (Figure 13B). This is similar to quenching efficiencies obtained in solution-phase assays (Dubertret et al., *Nature Biotech.* 19:365-370 (2001), which is hereby incorporated by reference in its entirety). Viewed another way, this corresponds to a 26-fold fluorescence enhancement for H1 in the presence of 1.38μM T1, or 20-fold enhancement in the presence of 2.29μM T2. Preliminary experiments designed to test the sensitivity of this technique indicated that this system could detect complementary DNA concentrations as low as 10 nM. However, this is by no means an optimized value. Based on recent measurements of the coverage of oligonucleotides on Au surfaces (Demers et al., *G. Anal. Chem.* 72:5535-5541 (2000), which is hereby incorporated by reference in its entirety), it is expected that optimization of the probe, site size, site density, and instrument design will improve detection to the fM level. It has also been observed that fluorescence unquenching of the chip is reversible, as washing the hybridized ("on") surface with unbuffered water restores it to a quenched ("off") state. Cycles of hybridization/washing results in a monotonic decrease in fluorescence intensity, presumably due to loss of probe hairpin from the Au surface.

As shown in Figure 14A-C, sensitivity was limited by a small background signal at 585 nm. This signal had a similar spectrum to that arising from just a pure Au film or a quartz coverslip, indicating that it was due to autofluorescence from the optical system.

Binding specificity (sequence selectivity) is obviously a significant measure of the utility of a diagnostic device or biosensor. To evaluate the extent to which the Au-immobilized probes retained their hybridization selectivity, the ability of equivalent concentrations of T1 and salmon sperm DNA (USB Corporation) to produce a signal when incubated with a H1-functionalized gold substrate were compared. As shown in Figure 14D, an approximately 26-fold increase in intensity (over background) was measured for the sample corresponding to the appropriate complementary DNA (curve c). In contrast, an equivalent concentration of salmon sperm DNA produces only a 4-fold increase of fluorescence intensity (curve f). This result indicates that DNA hairpins immobilized on a gold surface retain their ability to bind complementary DNA sequences selectively. That the salmon sperm DNA produces a net increase in intensity is not surprising, as a standard BLAST database search (Altschul et al., *Nucl. Acids Res.* 25:3389-3402 (1997), which is hereby incorporated by reference in its entirety) of the sequences T1 and T2 indicates that sequences homologous to portions of these are present in a variety of organisms.

While under laser illumination, the fluorescence intensity was observed to irreversibly decay with time, likely due to photobleaching of the dye molecule. For an excitation intensity of 600 W/cm^2, the signal intensity was reduced by a factor of 2 in a second. However, the rate of decay was linearly proportional to the excitation intensity for intensities in the range 6 to 600 W/cm^2. Thus, to avoid any ambiguities caused by the permanent photobleaching, all measurements were taken at intensities less than 20 W/cm^2. This is a lower intensity than is commonly employed by commercial microarray scanners; however, direct comparisons are difficult given the differences in scan times.

The above results demonstrate that fluorophore-tagged DNA hairpins attached to gold films can function as highly sensitive and selective sensors for oligonucleotides. For two distinct DNA hairpin sequences, binding by the complement caused an increase in signal by over a factor of 20, while non-specific sequences resulted in a minimal response.

Example 5 - Detecting of Mismatches Using Hairpin-Immobilized on Gold Film

A hairpin H3 (Figure 15A) was prepared by modification of hairpin H1. In particular, nucleotides 13-24 of H1 (SEQ ID NO: 1) were removed, forming H3 shown in Table 4 below. Similarly, target T3 was prepared by modification of target T1, specifically by removing nucleotides 25-37 from T1 (SEQ ID NO: 11), forming T3 shown in Table 4 below. Mismatch target T3M1 was prepared by modifying nt 6 of T3 (from C→G), as shown in Table 4 below.

Table 4: Sequences used in Example 5

Entry	SEQ ID NO:	Sequence
H3	13	(C6Thiol)-acacgctcatcaagctttaactcatagtgagcgtgt-Rhodamine
T3	14	acgctcactatgagttaaagcttg
T3M1	15	acgctgactatgagttaaagcttg

H3 and its respective complementary strands T3 (Figure 15B) and T3M1 (containing a single mismatch, Figure 15C) were obtained from a commercial supplier (Invitrogen Corporation, Carlsbad, California). H3 was bound to a gold surface in the same manner as described in Example 2 above.

Hybridization between H3 bound to the gold surface and either T3 or T3M1 was performed under the same conditions described in Example 3 above. The CCD images obtained illustrate the readily apparent differences in fluorescent intensity (compare Figures 16A-B) caused by the single base mismatch. The graph presented in Figure 16C represents the binned CCD images, which reflect a nearly five-fold reduction in fluorescence intensity (efficiency) for the target possessing a single mismatch. This example indicates that the present invention can readily be used to discriminate between a target nucleic acid having perfectly matched bases a nucleic acids possessing polymorphisms, such as single-nucleotide polymorphisms ("SNPs"). Therefore, the present invention is expected to be useful for purposes of analyzing genomic information for the presence of SNPs and other polymorphisms.

Example 6 - Preparation of DNA Hairpins Targeted to *Bacillus anthracis* DNA

Two DNA hairpins, HP1 and HP2 (Table 5 below), were designed to incorporate portions of the *Bacillus anthracis* partial *pag* gene, isolate IT-Carb3-6254 (Genbank Accession AJ413936, which is hereby incorporated by reference in its

entirety). These hairpins were designed according to the procedures described in co-pending U.S. Provisional Patent Application to Miller et al., entitled "Method of Identifying Hairpin DNA Probes By Partial Fold Analysis," filed concurrently with this application and expressly incorporated by reference in its entirety.

Table 5: Sequences used in Example 7

Entry	SEQ ID NO:	Sequence
HP1	6	(C6Thiol)-tttctttcaccatggatttctaatattcatgaaaagaaa-Rhodamine
HP2	5	(C6Thiol)-tcgttagtgttaggaaaaaatcaaacactcgcga- Rhodamine
TP1	16	tttcttttcatgaatattagaaatccatggtgaaagaaa
TP2	17	tcgcgagtgtttgatttttttcctaacactaacga

Basically, a partial gene sequence of the above-identified *pag* gene was obtained from the Genbank database and the secondary structure of an approximately 1000 nucleotide region was predicted using computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety). From this predicted structure, two naturally occurring hairpins were identified. One appeared at nt 668-706 of the *pag* sequence from Genbank Accession AJ413936. The other appeared at nt 1209-1241 of the *pag* sequence from Genbank Accession AJ413936.

Having identified these two sequences, these sequences were isolated from the larger sequence and subjected to a second structure prediction as above. The predicted structure of HP1 is characterized by a predicted free energy value of about -4.4 kcal/mol. The predicted structure of HP2 is characterized by a predicted free energy value of about -4.7 kcal/mol. In addition, these two hairpins are each within the size range of about 30-40 nucleotides. Having selected HP1 and HP2, a final structural prediction of the duplexes (HP1-TP1 and HP2-TP2) was carried out to determine the predicted free energy value for the duplexes. The duplex HP1-TP1 was predicted to have a free energy value of -43.2 kcal/mol and the duplex HP2-TP2 was predicted to have a free energy value of -42.6 kcal/mol. These values indicate that the hybridization between the hairpin and the target will be an energetically favorable process. A BLAST search was independently performed using the HP1 and HP2 sequences, the results indicating that only *pag* genes from other *Bacillus anthracis* isolates contain highly related nucleotide sequences.

Example 7 - Preparation and Testing of Sensor Chips Targeted to *Bacillus anthracis* DNA

(C6-thiol)-HP1-Rhodamine and (C6-thiol)-HP2-Rhodamine, as well as their respective complementary strands TP1 and TP2 (Figures 17A-B and Figure 18A-B), were obtained from a commercial supplier. A sensor chip for identifying *Bacillus anthracis* DNA, specifically DNA for the *pag* gene, was prepared by immobilizing HP1 and HP2 onto a gold-coated substrate in accordance with Example 2 above.

Hybridization between HP1 bound to the gold surface and TP1, as well as HP2 bound to the gold surface and TP2, was performed under the same conditions described above in Example 3 above. The CCD images obtained illustrate the readily apparent differences in fluorescent intensity for HP1-TP1 hybridization (compare Figures 17A-B) and for HP2-TP2 hybridization (compare Figures 18A-B). The graph presented in Figure 17C represents the binned CCD images, which illustrate a nearly 24-fold increase in fluorescence intensity upon target binding. Likewise, the graph present in Figure 18C represents the binned CCD images, which illustrate a nearly six-fold increase in fluorescence intensity upon target binding.

Together, these data indicate that the present invention can readily be used to prepare hairpin sensors capable of identifying the presence of target DNA in a sample. Although T3 was a synthetic nucleic acid representative of *Bacillus anthracis pag* gene, it is expect that the DNA from samples containing *Bacillus anthracis* should produce similar results given the specificity of the selected hairpin.

Example 8 - Preparation of DNA Hairpins Targeted to *Staphylococcus aureus* DNA

Two DNA hairpins, AH2 and BH2 (Table 6 below), were designed to incorporate portions of the *Staphylococcus aureus* genome (Genbank Accession AP003131, which is hereby incorporated by reference in its entirety). These hairpins were designed according to the procedures described in co-pending U.S. Provisional Patent Application to Miller et al., entitled "Method of Identifying Hairpin DNA Probes By Partial Fold Analysis," filed concurrently with this application and expressly incorporated by reference in its entirety.

Table 6: Sequences used in Example 9

Entry	SEQ ID NO:	Sequence
AH2	9	(C6Thiol)-cgataatatgatgcctaggcagaaatattatcg-Rhodamine
BH2	10	(C6Thiol)-tatcaataataaacgaataggggtgttaatattgata-CY5
AH2-C	18	cgataatatttctgcctaggcatcatattatcg
BH2-C	19	tatcaatattaacacccctattcgtttattattgata

Basically, a segment of the complete *Staphylococcus aureus* genome was obtained from the Genbank database and the secondary structure of the obtained segment was predicted using computer program RNAStructure version 3.7 (Mathews et al., *J. Mol. Biol.* 288:911-940 (1999), which is hereby incorporated by reference in its entirety). From this predicted structure, two naturally occurring hairpins were identified, one corresponding to AH2 and the other corresponding to BH2.

Having identified these two sequences, these sequences were isolated from the larger sequence and subjected to a second structure prediction as above. The predicted structure of AH2 is characterized by a predicted free energy value of about -6.1 kcal/mol (Figure 24). The predicted structure of BH2 is characterized by a predicted free energy value of about -3.5 kcal/mol (Figure 25). In addition, these two hairpins are each within the size range of about 30-40 nucleotides. Having selected AH2 and BH2, a final structural prediction of the duplexes (AH2-AH2C and BH2-BH2C) was carried out to determine the predicted free energy value for the duplexes. The duplex AH2-AH2C was predicted to have a free energy value of -38.3 kcal/mol and the duplex BH2-BH2C was predicted to have a free energy value of – 39.0 kcal/mol. These values indicate that the hybridization between the hairpin and the target will be an energetically favorable process. A BLAST search was independently performed using the AH2 and BH2 sequences, the results indicating that only segments of the *Staphylococcus aureus* genome contain highly related nucleotide sequences.

Example 9 - Preparation of Single Chips Containing Two Hairpins That Identify Distinct Targets

(C6-thiol)-AH2-Rhodamine and its complementary strand AH2-C was obtained from Midland Certified Reagent Co. (Midland, Texas). (C6-thiol)-BH2-CY5 and its complementary strands BH2-C was obtained from Integrated DNA Technologies, Inc. (Carlsbad, California).

Glass slides were cleaned with piranha etch solution (4:1 concentrated H_2SO_4/30% H_2O_2) overnight at room temperature, and then rinsed with Nanopure water. Metal deposition was performed at a rate of 0.2 nm/s using Denton Vacuum Evaporator (DV-502A). First, a chromium adhesion layer of 7 nm was coated on the glass, followed by 100 nm thick gold films. Before use, the gold substrates were annealed at 200°C for 4 hour and cleaned with piranha solution for 0.5-1 hr.

Two sensor chips for identifying *Staphylococcus aureus* DNA were prepared by immobilizing AH2-Rhodamine and BH2-CY5 onto the same gold-coated substrate. AH2-Rhodamine and BH2-CY5 were mixed together in 1:1 ratio. The gold substrate was soaked in a mixture solution of hairpin oligonucleotide and mercaptopropanol at a ratio of 1 to 10 for self-assembly. Two hours later, the substrate was thoroughly rinsed with hot water (90°C or higher) to get rid of the non-bonded DNA and then was immersed in 0.5 M NaCl buffer (20 mM Cacodylic acid, 0.5 mM EDTA, 0.5 M NaCl, pH = 7) for 1-2 hours. Next, two substrates carrying the mixed probes were incubated in the target solutions for AH2 and BH2, respectively, for hybridization at room temperature for 16 hours under the same buffer conditions.

Fluorescence measurement was performed on a Nikon inverted fluorescence microscope equipped with a liquid nitrogen cooled CCD. A CW laser (Millenia, Spectra-Physics) was used for excitation at 532 nm. The beam passed through a high-pass dichroic mirror and a notch filter before it reached the sample. The DNA chip was inverted on a clean cover slip on top of a 60× air objective. Fluorescence emission was collected by the same objective and directed through a long pass filter (570 nm) to a CCD. To track the fluorescence of a certain area, a pattern was scratched on the gold so that exactly the same area could be examined before and after hybridization for comparison. At least four areas at different positions of the gold were chosen for each sample during fluorescence measurement. Under laser illumination, the images were recorded by the CCD camera at 5s integration time and the spectra were recorded at 30s integration time. For each spot, an image was taken, a spectrum 550nm to 630nm (for rhodamine) and then at 620nm to 700nm (for CY5).

For chip AB-3, the increase of Rhodamine is higher than that of Cy5, so the fluorescence increase of the chip is mainly due to probe AH2-AH2C hybridization, rather than BH2-AH2C hybridization (Figures 19A-B; Figures 20A-B).

The increase in fluorescence emission for chip AB-3 is about 3.6-fold (Figure 20C). For chip AB-4, the increase of Cy5 is higher than that of Rhodamine, so the fluorescence increase of the chip is mainly due to probe BH2-BH2C hybridization, rather than AH2-BH2C hybridization (Figures 19C-D; Figures 20D-E). The increase in fluorescence emission for chip AB-4 is about 1.6 fold (Figure 20F).

Example 10 - Preparation of Hairpin Probe Labeled with CdSe Nanocrystal as Fluorophore

CdSe nanocrystals capped with ZnS were dissolved in hexane as stock solution. Two ml of the nanocrystal stock solution was washed with methanol three times. The washed nanocrystals were then introduced into 0.5ml N,N-dimethylformamide (DMF), followed by adding 12μL dihydrolipoic acid (DHLA). The reaction was allowed to proceed overnight under nitrogen and in dark. Thereafter, the nanocrystals were precipitated and washed twice with acetonitrile. About 1.79 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added to the nanocrystal-acetonitrile solution. After about four hours, the nanocrystals were washed again with acetonitrile. An aqueous solution of oligonucleotide hairpins (having the 3′ end modified with an amine group) was mixed with nanocrystals under mild stirring and at room temperature. About 24 hours later, the labeled oligonucleotide hairpins were washed in methanol and then dissolved in water or mild saline buffer solution for subsequent use.

It is expected that the nanocrystal-labeled hairpin will afford orders of magnitude more photostability for the sensor chips as compared to the dye-labeled hairpins described in the preceding examples.

In addition to the foregoing examples, it should be appreciated that additional design considerations can be implemented. For example, sensitivity of the sensor chip can be further optimized through surface enhancement provided by roughened quenching (e.g., metal) substrates (Cao et al., *Science* 297:1536-1540 (2002); Haes et al., *J. Am. Chem. Soc.* 124:10596-10604 (2002), each of which is hereby incorporated by reference in its entirety). Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can

be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.